Purification and Characterization of Milk-clotting Enzyme from *Solanum dubium* Seeds

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ABSTRACT

This research was carried out to purify and characterize a milk-clotting enzyme from *Solanum dubium* seeds and to investigate the efficiency of the purified enzyme as a rennet substitute in cheese making. A novel milk-clotting serine protease was purified to 6.7 folds and 35% recovery from *S. dubium* seeds by combination of $(NH_4)_2SO_4$ precipitation, cation-exchange and gel filtration chromatographies. The molecular mass of the enzyme was 66 kDa as estimated by gel filtration and SDS-PAGE. The purified enzyme was a chymotrypsin-like serine protease with p*I* value of 9.3, acts optimally at pH 11.0 and stable over a wide range of pH (4.0-11.0). The purified enzyme was also thermostable protease with stability of up to 60°C for one hour and acts optimally at 70°C for 30 min. When compared with other plant enzymes, *S. dubium* enzyme had higher ratio of milk-clotting activity /proteolytic activity. The most striking property of *S. dubium* milk-clotting serine protease, among all reported plant serine proteases, was its high stability under several conditions (pH, temperature, organic solvents and denaturants). The enzyme exhibited broad specificity toward bovine whole casein and its components (α-, β- and κ-caseins). Unlike the other milk clotting enzymes, *S. dubium* milk-clotting protease degraded β-casein faster than α- casein. The high stability of the enzyme under various conditions, in accordance with its milk-clotting ability, could therefore, pave the way for its uses in cheese industry and biotechnology.

Key words: Casein hydrolysis; enzyme activity; milk-clotting, *Solanum dubium*

INTRODUCTION

Cheese is a dairy product that has played a key role in human nutrition for centuries. The broad range of different cheeses available is based mainly on regional conditions and production technology, which has been repeatedly adapted and optimized. The main objective has always been, and still is, to convert milk, which is perishable, into a product with a longer shelf life whilst preserving its nutrients. Coagulation of milk is

the basic step in the manufacture of all cheeses. Calf rennet, which contains chymosin (EC 3.4.23.4) as the main enzyme component, has been the most widely used milk-clotting enzyme preparation. Increasing world cheese production and consumption besides the increase of calf rennet's price, along with a reduced supply of calf rennet, has led to a systematic investigation for new rennet substitutes. Much research interest has been directed towards discovering a milk-clotting enzyme which would satisfactorily replace calf rennet in cheese manufacture, and numerous enzyme preparations of animal, microbial, and plant origin have been studied (Fox 1969). Consumer constraints on the use of rennets have led to a growing interest in vegetable coagulants. The use of animal rennet may be limited for religious reasons (e.g. Judaism and Islam), diet (vegetarianism), or being against genetically engineered foods (e.g. Germany and the Netherlands refused the use of recombinant chymosin) (Roseiro *et al.*, 2003).

The incidence of bovine spongiform encephalopathy (BSE) has reduced both supply and demand for bovine rennet (Roseiro *et al.*, 2003). Microbial rennets produced by genetically engineered bacteria have proven suitable substitutes for animal rennet, but increasing attention has been directed toward natural rennet extracted from plants (Shah *et al*., 2013). Unfortunately, most of these plant rennets were found unsuitable because they produce extremely bitter cheeses. An exception to this general rule is represented by the aqueous extract of *Cynara cardunculus* flowers containing two aspartic acid-type proteases, named cardosin A and B (Verissimo *et al.*, 1995), which have been used for years for the manufacture of sheep milk cheese in several areas of Portugal and Spain. Studies on the specificity of cardosin A showed that it cleaves only the Phe_{105}-Met_{106} bond like chymosin, the proteolytic coefficient being of the same order of magnitude (Macedo *et al.*, 1993). Cardosin B, instead, is similar, in term of specificity and activity, to pepsin. The application of vegetable rennet for cheese making could improve the nutrition of the populations, where restrictions are imposed against the use of animal rennet particularly in developing countries.

Gubbein (*S. dubium*), a major problem for many farmers in Sudan, is a noxious weed belonging to the plant family *Solanaceae* that grows in vast areas of Sudan. It is a perennial plant that flourishes during the rainy season (typically starting in June-August in Sudan) and usually bears fruits about January with green fruits, which become yellow when fully ripe. Fruits usually dry on the stem their thorny surface causes them to adhere to grazing animals and facilitates seed dissemination. Animals do not eat *S. dubium* because of its bitter taste and thorny leaves (Yousif *et al.*, 1996). Dairy farmers in some parts of the Sudan use the berries of *S. dubium* to make a type of white soft cheese known as *Jibna beida* (white cheese) from goat and sheep milk. About 10-15 *S. dubium* berries are crushed in a cup half-filled with water (about 250 mL). After 5 min, the extract is strained and added to about 20kg of salted milk. Milk coagulation takes about 2 h. The curd is cut, dipped for 30 min; the whey is drained and the curd is pressed. The resultant cheese has a slight bitter taste and a fragile crumbly texture. The bitterness of *Jibna beida*

cheese is probably a result of the presence of some alkaloids or unspecific proteolytic activity of enzymes obtained from the berries. It may be possible to reduce bitterness by using a purified enzyme and by using optimum extract concentration (Yousif *et al.*, 1996). Many studies were carried out to determine the plant as a source of milk clotting enzyme (Mohamed and Habbani, 1996; Osman, 2001). However, detailed and accurate studies to determine the chemical characteristics of the purified enzyme as a milk coagulant for cheese making were not yet carried out. Therefore, the main aim of the present study was the purification and characterization of milk clotting enzyme from the seeds of *S. dubium*.

MATERIALS AND METHODS

Materials

Solanum dubium fruits were obtained from Shambat area, Khartoum North, Sudan. The fruits were sun dried and hand crushed to obtain the seeds. All chemicals were pure grade chemicals from either Wako (Wako pure chemical, Japan) or Sigma (Sigma-Aldrich, Japan), skimmed milk powder, Hammerstein casein, phenylmethylsulphonyl fluoride [PMSF], Chymostatin, Soybean trypsin inhibitor were from Wako (Wako pure chemical, Japan). Azocasein, AEBSF (4-(2-aminoethyl) benzene sulfonyl fluoride hydrochloride, were from Sigma. Standard protein for gel filtration and SDS-PAGE were from Bio-Rad laboratories (Hercules, CA, USA). CM-Toyopearl was from (Tosoh, Tokyo, Japan) and Superdex 75 was from Amersham (Amersham Bioscience, Uppsala, Sweden).

Methods

Enzyme extraction: *Solanum dubium* seeds (50 g) were coarsely milled in a mortar and extracted with 500 mL of 5% NaCl in sodium acetate buffer, pH 5.0, with stirring at 4°C for1h. The extract was filtrated through cheesecloth and centrifuged at 12,000 rpm for 20 min. The supernatant was dialyzed overnight at 4°C with 0.1 M sodium acetate buffer, pH 5.0. Milk clotting and protease activities were routinely determined.

Ammonium sulfate fractionation: The supernatant (510 ml) from the above step was brought to 35% saturation with the gradual addition of solid ammonium sulfate and allowed to stand on ice for 30min. The resulting precipitate was collected by centrifugation at 12000 rpm (HIMAC, type SCR 18B and CR 20B2, Hitachi Koki Co., Ltd, Tokyo, Japan) for 20 min at 4^{o}C and dissolved in 50 mM sodium acetate buffer, pH 5.0. The above step was repeated twice to obtain 55 and 80% saturation. The supernatant of each step was dialyzed with 50 mM sodium acetate buffer, pH 5.0, for 24 h with frequent changes of buffer. After dialysis, the solution was centrifuged to remove any solid particles and then the protein concentration and activity were measured.

Ion-exchange chromatography (CM-Toyopearl): The active fraction (65 ml) obtained above was further purified by passing through CM-Toyopearl column (2.6cm X 85cm) equilibrated with 50 mM sodium acetate buffer, pH 5.0. The column was washed three times with the same buffer until the absorbance at A_{280} nm reached zero. Then, elution was carried out using a linear gradient of 0.0 to 0.5 M KCl in 50 mM sodium acetate buffer, pH 5.0. Fractions of 18 ml each were collected at a rate of 3.0 mL/min, and analyzed for enzyme activity and protein concentration. The partially purified enzyme (320 ml) from the above step was further purified using ammonium sulfate (2^{nd} fractionation) with saturation ranged from 35% to 80% (5% interval). Then, the most active fractions in the ranges of 40%~45%, 45%~50%, and 50%~ 55% were pooled and combined as one fraction (40%~55%) and dialyzed overnight at 4^{o}C with 50 mM sodium acetate buffer, pH 5.0. Thereafter, the pooled fraction was gel filtered using Superdex 75.

Gel filtration chromatography (Superdex 75): The pooled fractions of the above step were concentrated using an Amicon ultra membrane with 10000 Dalton cut off (Millipore, Carrigtwohil, Co. Cork, Ireland). The concentrated solution (3.0ml) was applied to a Superdex 75 column (1.6cmX58cm) equilibrated with 50 mM sodium acetate buffer, pH 5.0, containing 0.1 M KCl. Elution was carried out at a flow rate of 1.0 ml/ min (3.0ml/ tube). Active fractions were pooled and stored at–20°C for further analysis.

Determination of milk-clotting activity: Milk-clotting activity was determined according to the methods described by Arima *et al.* (1970) with slight modification. The substrate (10% skimmed milk in 0.01 M $CaCl_2$) was prepared and the pH was adjusted to 6.5. The substrate (2.0 ml) was pre-incubated for 5 min at 37°C, and 0.2 ml of enzyme extract was then added, and the curd formation was observed (at 37°C) while manually rotating the test tube from time to time. The end point was recorded when discrete particles were discernible. One milk-clotting unit was defined as the amount of enzyme that clots 10 ml of the substrate in 40 min.

MCA (U/ml) = (2400⁄ clotting time (sec)) × Dilution factor

Determination of protease activity: Protease activity was determined by the colorimetric assay using azocasein as a substrate (Sarath, 1989). About 0.15 ml enzyme solution was added to 0.25 ml of 1% azocasein in 50 mM Tris-HCl buffer, pH 8.0, mixed gently and incubated at 30°C for 30 min. The reaction was stopped by adding 1.2 ml of 5% TCA, the mixture was incubated at room temperature for 30 min, then centrifuged at 10,300 rpm for 10 min at 4°C. One ml of the supernatant was mixed with 1.0 ml of 1.0 M NaOH and kept for 10 min at room temperature for color development. A blank was prepared in the same way, but with the addition of TCA to the enzyme before the substrate. The absorbance was read at 440 nm. One unit of the enzyme activity was

defined as the amount of the enzyme that increased the absorbance at 440 nm by 1.0/min under the assay conditions.

Electrophoresis and activity staining: Homogeneity and intactness as well as molecular mass determination of the enzyme, during and after purification, were determined using SDS-PAGE (Laemmli, 1970). The purified enzyme was inactivated to avoid autolysis. The gels were stained with 0.1% Coomassie brilliant blue R-250. The molecular weight of the purified enzyme was extrapolated from the plot of log molecular weight vs electrophoretic mobility of markers.

For activity staining (zymography), the electrophoresis of the purified enzyme was carried as described above except that the sample was treated with buffer containing 2% SDS without boiling to keep the enzyme active. After electrophoresis, the gel was washed with 2.5% Triton X-100 for 30 min with shaking to remove SDS, and then washed three times with distilled water. The gel was then immersed in 50 ml of 2% casein in Tris-HCl buffer, pH 7.5, for 30 min at 4°C, in order to allow the substrate to diffuse into the gel at reduced enzyme activity (Garcia *et al.*, 1993). Then, the gel was incubated at 25°C for 60 min to digest the substrate. Thereafter, the gel was washed with distilled water, and immediately fixed and stained.

Hydrolysis of casein: Hydrolysis of bovine casein was carried out according to the method described by Sousa and Malcata (1998). Bovine casein was dissolved in 100 mM phosphate buffer, pH 6.5, to a final concentration of 10 mg/ml. The enzyme (0.025 U/ml) was added to the casein substrates at a ratio of 0.5 mL to 10 mL of (v/v), and reaction was allowed to proceed at 30°C. Aliquots were taken periodically, and the reaction was quenched by heating at 100°C for 5 min. Samples were analyzed by SDS-PAGE.

Effect of pH on the enzyme activity and stability: The optimum pH of the enzyme was determined by measuring azocasein hydrolyzing activity at 30°C for 30 min in a pH range of 5.0 to12.8. The buffers used were; 50 mM citrate-sodium phosphate (pH 5.0–6.0), 50 mM Tris-HCl (pH 6.0-8.0), and 50 mM glycine (pH 8.0–12.8 adjusted with NaOH). Suitable controls at the respective pH values were also set up, comprising the buffered substrate without enzyme. The pH stability of the enzyme was determined by measuring the remaining activity after incubation at 30°C for 1h, and 25 h or at 60°C for 30 min in different buffers (50 mM sodium dihydrogen phosphate/ NaOH buffer between pH 3.0 and 8.0, and 50 mM glycine/ NaOH between pH 8.0 and 12.8).

Effect of temperature on the enzyme activity and stability: Optimum temperature for the enzyme activity was studied using azocasein as substrate. The substrate was pre-incubated at specified temperatures in a range of 20°C- 90°C for 10 min. Then the enzyme was added, the reaction was proceeded at the same temperature for 30 min. and a

control tube was incubated without the enzyme at each temperature. Stability was tested by incubating the enzyme in 50 mM Tris-HCl buffer, pH 8.0, at different temperatures (20- 90°C) for 30 min. The remaining activity was measured at 30°C for 30 min using azocasein as a substrate and was expressed as a percentage of the control.

Stability of the enzyme under various denaturants and organic solvents: The enzyme stability in the presence of chemical denaturants such as urea, SDS, and Guanidine hydrochloride or organic solvents such as methanol, and acetonitrile was studied. The enzyme (12µg) was incubated with 3.0 M GuHCl, 3.0 M NaCl, 7.0 M urea, 2% SDS, 50% methanol, or 50% acetonitrile at 30°C for 1 h, and then the residual activity was determined using 1% azocasein in 50 mM Tris-HCl buffer, pH 8.0, at 30°C for 30 min.

RESULTS AND DISCUSSION

Enzyme purification

Ammonium sulfate precipitation was performed as the first purification step of *S. dubium* milk-clotting protease. The results (Table 1) showed that the (35%-55%) saturation fraction had the highest milk-clotting and specific activities. By applying 35%-55% ammonium sulfate precipitation as the first purification step, over 86% of the total protease in the crude extract was salted out. This procedure not only facilitated the effective removal of the brown-colored materials (existing) in the crude extract, it also concentrated the enzyme preparation to a workable volume that could be loaded onto CM-Toyopearl column. As shown in Figure 1, the loaded sample was separated into two protein peaks. The second peak contained the milk-clotting activity, which was eluted with 0.2 - 0.3 M KCl in 50 mM sodium acetate buffer, pH 5.0. In this step, the clotting enzyme was purified to 2.7-fold with 64.3% yield and a specific activity of 82.1U/mg proteins. Gel filtration chromatography was used as a polishing step in the purification of milk-clotting enzyme from *S. dubium* seeds, in which the enzyme was eluted as one active peak (Fig. 1). Our results are in conformity with the results obtained during the purification of serine protease from *Cucumis trigonus* Roxburghi, in which after three steps of purification including anion-exchange, ammonium sulfate and cation exchange chromatography a similar purification level was obtained (Asif-Ullah *et al.*, 2006). It worth to note that recovery obtained during the purification course of this study was higher than that of the aforementioned study, which indicated that the milk clotting enzyme constitutes most of the protein in *S. dubium* seeds. Similarly, many serine proteases were purified to the same level of purification (Kocabiyik and Ozedmir 2006; Joo and Chang 2005). On the other hand, a milk-clotting aspartic protease was also purified to the same purification level from *Rhizomucor miehei* (Preetha and Boopathy, 1997). A simple purification procedure has been developed to simultaneously purify milk-clotting enzyme from *S. dubium* seeds. Such an economic purification procedure

combined with the easy availability of the plant seeds makes large scale preparation of the enzyme possible, allowing a broad study of its various aspects and hence probable applications.

Table 1. Purification of milk clotting enzyme from *S. dubium* seeds

Purification means	Protein (mg)	Total activity (Units)	Specific activity (Units/mg)	Yield (%)	Purification (Fold)
Crude extract	2.1×10^3	65.4×10^3	30.5	100.0	1.0
Ammonium sulfate (35%-55%)	968.5	56.4×10^3	58.2	86.2	1.9
Ion exchange chromatography	512.0	42.0×10^3	82.1	64.3	2.7
Ammonium sulfate (40%-55%)	444.6	40.7×10^3	91.6	62.3	3.0
Gel filtration chromatography	111.5	22.9×10^3	205.4	35.0	6.7

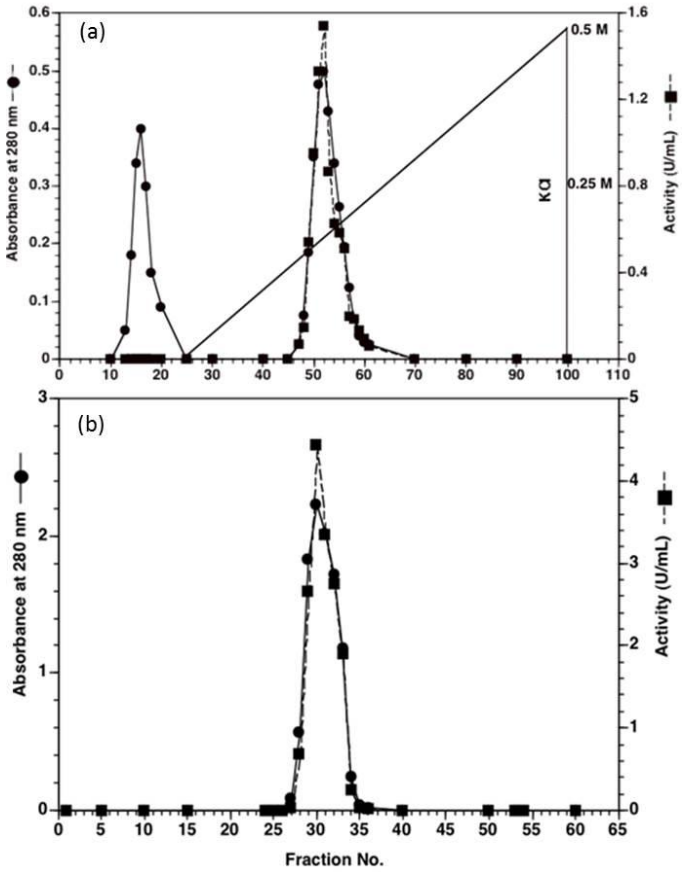

Fig. 1. Elution profile of *S. dubium* milk clotting enzyme on (a) cation exchange resin. The CM-Toyopearl column (2.6 cm X 85 cm) was pre-equilibrated with 50 mM sodium acetate buffer, pH 5.0. Then the column was washed with the same buffer and eluted at a flow rate of 3.0 ml/min with a linear gradient of 0.0- 0.5M KCl in the same buffer. (b) Gel filtration chromatography on a Superdex 75 column. The concentrated sample (3.0 ml) after second ammonium sulfate fractionation was loaded onto a Superdex 75 column (1.6 cmX58 cm), and the column was washed, and eluted with 0.1 M KCl in 50 mM sodium acetate buffer, pH 5.0, at a flow rate of 1.0 ml/ min. All fractions were assayed for protein and milk-clotting activity.

Physicochemical properties of the purified enzyme

Polyacrylamide gel electrophoresis under reducing conditions (SDS-PAGE) was used to check the purity and to determine the molecular mass of the enzyme. The purified enzyme showed a single band with a molecular mass of 66 kDa on SDS-PAGE under reducing and non-reducing condition (Fig 2). A single band on SDS-PAGE, Native-PAGE electrophoretograms and one protein peak on gel filtration revealed high purity of the enzyme. The molecular mass of *Solanum dubium* milk-clotting enzyme is similar to that of well-known plant serine protease Cucumisin (Yamagata *et al.,* 1989), and with other cucumisin-like serine proteases (Asif-Ullah *et al.,* 2006; Curroto *et al.,* 1989; Uchikoba *et al.,* 1998; Rudenskaya *et al.,* 1998). The molecular mass of the purified enzyme was also

similar to those of subtilisin-like endoproteases from tomato plant (Messdaghi and Dietz, 2000; Tornero *et al.,* 1996). The molecular masses of plant serine proteases so far known varied from 19 to 110 kDa, however the majority lies between 60 and 80 kDa (Antao and Malcata 2005). It is worth to note that *S. dubium* serine protease was irreversibly inactivated by PMSF for 30 min before SDS-PAGE analysis. It was partially autolyzed under the routine conditions for SDS-PAGE involving boiling the enzyme sample. This indicates that the purified enzyme is liable to autolyze if the native structure of the enzyme is partially changed by a denaturing agent to induce more or less unfolding of the enzyme. This might be the reason why SDS-PAGE electrophoretograms of the enzyme sample, sometimes, showed several small bands when the enzyme had not been completely inactivated before the analysis. Similar observations were reported on SDS-PAGE analysis of plant serine proteases Milin (Yadav *et al.,* 2006) and Cucumisin (Yamagata *et al*., 1989), and on SDS-PAGE analysis of the alkaline serine protease of *Bacillus* KSM-K16 (Kobayashi *et al.,* 1996) and *Bacillus sphaericus* (Singh *et al.,* 2001). Zymogram activity staining also showed one clear zone of proteolytic activity against a blue background. Although the zymogram gel contained 0.1% SDS and the sample was treated with 1% SDS, the enzyme still displayed activity, indicating that the *S. dubium* milk-clotting enzyme is resistance to SDS denaturing. As recently reported, SDS resistance is a property often associated with heat-stable proteases of thermo-stable *archaea* and *bacteria* (Joo and Chang, 2005). Some plant serine proteases were also reported as SDS resistance, example of this is Cucumisin (Yamagata *et al.,* 1989). It was also found that the enzyme had an isoelectric point (p*I* 9.3) indicating that the enzyme is a basic protein. In agreement with our result, Noda *et al.* (1994) found basic isoelctric point (p*I* 9.5) for plant serine protease from *Cucumis melo* L.

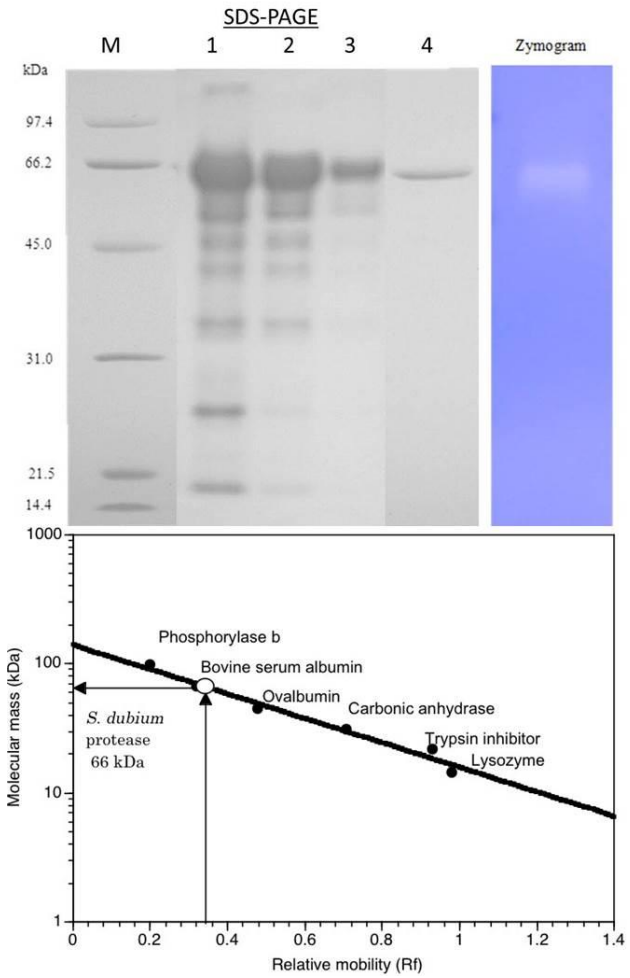

Fig. 2. Upper figure represent SDS-PAGE and Activity staining (Zymogram) milk clotting enzyme from *S. dubium* seeds. Lane M; molecular weight standards from high molecular weight descending; Phosphorylase b, Bovine Serum Albumin, Ovalbumin, Carbonic anhydrase, Soybean trypsin inhibitor, and Lysozyme. Lane 1; crude extract, lane 2; ammonium sulfate fractionation, lane 3; ion exchange chromatography, and lane 4; purified enzyme after gel filtration chromatography. Lower figure showed estimation of molecular mass of the purified enzyme by SDS-PAGE.

Enzyme activity and stability

Coagulants should not be sensitive to variations in milk composition and pH, since the use of highly pH-sensitive rennet can lead to reduced yields and defective cheese due to soft coagulum at cutting (Harboe and Budtz, 1999). *S. dubium* milk-clotting enzyme is stable under wide range of pH (4.0~11.0), and act optimally at pH11.0 (Fig. 3). The isolated enzyme is more stable at basic pH, and its stability is more comparable to those of Cucumisin-like serine proteases from *Cucumis trigonus* Roxburghi, *Cucumis melo* L. var. Prince, *Euphorbia milii*, and *trichosantus kirrilowi* A (Asif-Ullah *et al.*, 2005; Yamagata *et al.*, 1989; Yadav *et al.*, 2006; and Uchikoba *et al.*, 1990), respectively. These characteristics are important, because most enzymes are catalytically unstable at alkaline pH values, thus limiting their usefulness as cheese making coagulants (Lamas *et al.*, 2001).

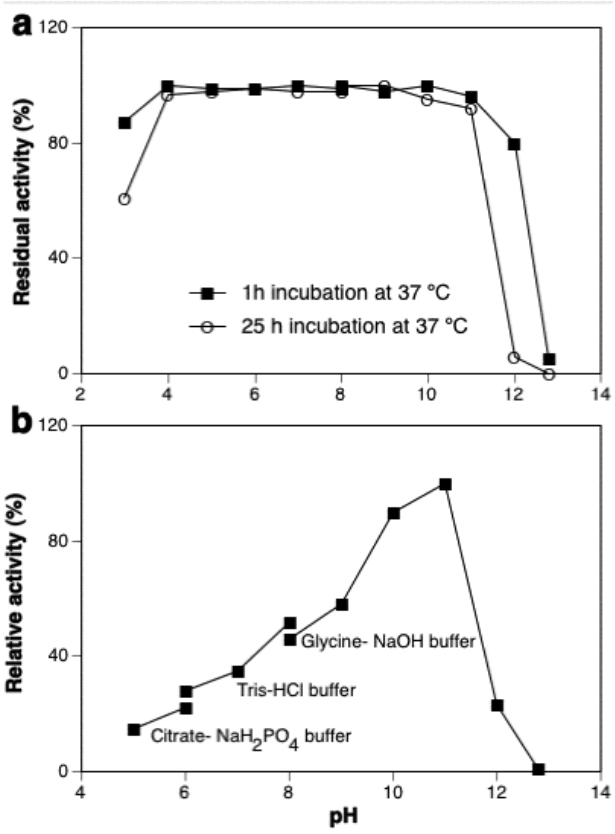

Fig 3. Effect of pH and temperature on the activity and stability of the purified enzyme. (a) pH stability of the purified enzyme. The enzyme (12µg) was incubated at various pH values for 1 or 25 h at 37°C. Residual activity was measured after 30 min at 30°C and pH 8.0. (b) Effects of pH on the activity of the purified protease. Enzyme activity was determined using 1% azocasein as substrate at various ranges of pH 5.0–12.8 at 30°C for 30 min. The buffer used for pH 5.0- 6.0 was 50 mM acetate/ Phosphate, for pH 6.0–8.0 it was 50 mM Tris-HCl, and for pH 8.0–12.5 it was 50 mM glycine-NaOH.

Results also revealed that *S. dubium* milk clotting enzyme had high optimum temperature as well as temperature stability (Fig. 4). The temperature optimum for *S. dubium* protease activity was found to be 70°C, which is in agreement with those of subtilisin/ cucmisin like plant serine proteases from *Cucumis trigonus* Roxburghi (Asif-Ullah *et al.*, 2006) and *Cucumis melo* L. var. Prince (Yamagata *et al.*, 1989). With regard to the stability of *S. dubium* serine protease at high temperature, it behaved like milk-clotting enzymes extracted from other vegetable sources and from microorganisms. However, *S. dubium* milk-clotting enzyme was characterized by high thermal resistance as compared to calf rennet, since calf rennet reached its maximum activity at 45°C, followed by a sharp decline when the temperature exceeded 50°C (Shehata *et al.*, 1996).

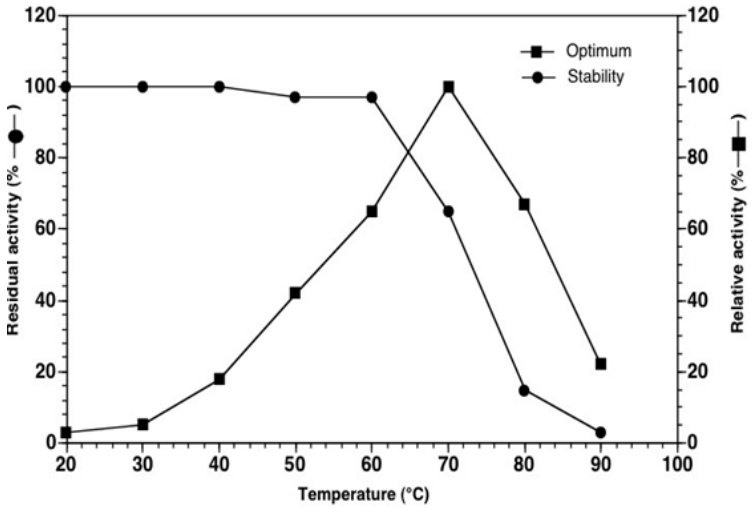

Fig. 4. Effects of temperature on activity and stability of the purified enzyme. For optimum temperature, the enzyme activity was measured using 1% azocasein in Tris-HCl buffer, pH 8.0, at various temperatures ranging from 20 to 90°C for 30 min. For temperature stability, the purified protease (12µg) was pre-incubated at various temperatures (20 to 90°C) for 1h, and the residual activity was measured using1% azocasein in Tris-HCl buffer, pH 8.0, at 30°C for 30 min.

In order to identify the class of isolated protease, the effects of different protease inhibitors were examined. The strongest inhibition was observed with PMSF a general inhibitor for serine proteases, in which the clear activity zone was completely disappeared (Fig. 5, lane 6); indicating that the purified enzyme is belonging to the serine protease family. The inhibition profile of *S. dubium* milk-clotting serine protease are consistent to those reported for bamboo serine protease (Arima *et al.*, 2000) and Cucumisin (Uchikoba *et al.*, 1995). The strong inhibition by PMSF was also reported for some plant serine proteases; Cucumisin-like protease from latex of *Euphorbia supina* (Arima *et al.*, 2000), subtilisin-like protease from *Cucumis trigonus* Roxburghi (Asif-Ullah *et al.*, 2006), and Milin (Yadav *et al.*, 2006).

Solanum dubium milk clotting enzymes exhibited remarkable stability under various conditions. Complete retention of proteolytic activity of the enzyme was observed in 7.0 M urea, 3.0 M GuHCl (Guanidine hydrochloride), and 3.0 M NaCl for 1 h incubation (Table 2). Moreover, the enzyme retained more than 95% of its activity when incubated for 1 h in methanol (50%) and acetonitrile (50%), and about 70% in 2% SDS. The stability of proteins and enzymes is usually the factor that limits their usefulness in many applications. The most striking property of *S. dubium* milk-clotting serine protease is its high stability with respect to pH, temperature, denaturants and organic solvents. These are the advantage of *S. dubium* protease among all the reported plant serine proteases. An exception is milin, which showed high stability under different conditions (Yadav *et al.*, 2006). High stability in GuHCl (4 M), and urea (8 M) have been reported for some plant cysteine proteases (Dubey and Jagannadham, 2003; Kundu *et al.*, 2000). The high stability of *S. dubium* milk-clotting serine protease against pH, temperature, denaturant, and organic solvent may make it a valuable tool for food industries and biotechnology, where other proteases fail to work in these extreme conditions.

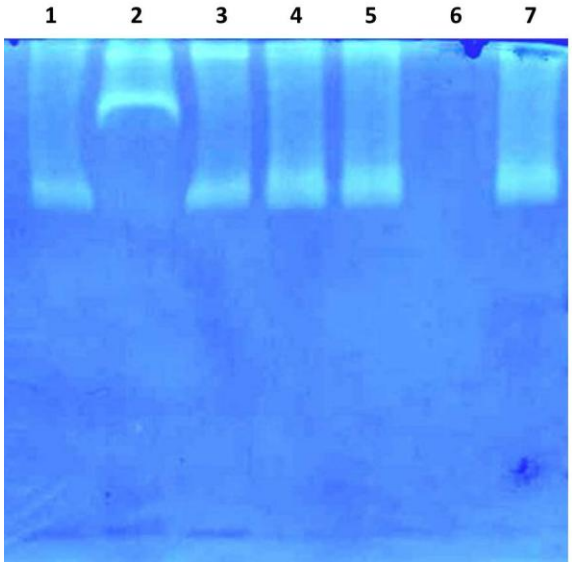

Fig. 5. Effect of different inhibitors on the activity of the purified enzyme. The enzyme (3 ug) was incubated for 30 min at 37°C in the presences of various chemical compounds. Lane 1, Dithiothreitol (DTT, 20 mM), Lane 2; Mercaptoethanol (2%), lane 3; Sodium monododecyl sulfate (SDS, 2%), lane 4; Urea (7.0 M), lane 5; Iodoacetamide (2.5 %), lane 6; Phenylmethanesulfonyl fluoride (PMSF, 40 mM), and lane 7; Control.

Table 2. Stability of purified enzyme under various denaturant and organic solvents

Condition	Relative activity (%)
Urea (7.0 M)	100
GnHCl (3.0M)	100
NaCl (3.0 M)	100
Acetonitrile (50%)	98
Methanol (50%)	96
SDS (2.0 %)	70

The purified enzyme (12µg) was incubated with different organic solvents and denaturants at the indicated concentration for 1 h at 30°C. Remaining activity was measured using 1% azocasein in 50 mM sodium acetate buffer, pH 5.0, as substrate at 30°C for 30 min.

Potentiality of using the purified enzyme in cheese making

Table 3 summarizes the ratio of milk clotting activity to proteolytic activity of *S. dubium* enzyme in comparison with those of some commercial rennets (Arima *et al.*, 1970). It was found that the ratio of milk clotting activity to proteolytic activities of *S. dubium* enzyme was comparable to those of Calf rennet, *Mucor* rennet and *Endothia parasitica* rennet. The ratio of milk clotting activity to proteolytic activity is a useful indicator of the protease efficiency to be used as a coagulant for cheese making (Arima *et al.*, 1970). Furthermore, *S. dubium* enzyme greatly coagulated skimmed milk and formed a white curd (Fig. 6). The capacity of the isolated enzyme to produce milk curds, beside its high ratio of milk clotting to proteolytic activity, could make it useful as a new milk coagulants, although, more studies about quality of both milk curds and the cheese formed should be carried out to confirm its usefulness in the dairy industry.

Table 3. Ratio of milk clotting activity/ proteolytic activity

Enzyme	Clotting activity (Units/mL)	Proteolytic activity (OD 660 nm)	Ratio (Units/OD 660 nm
Rennet	249.6	0.05	4992
Mucor rennet	551	0.11	4650
Endothia parstica enzyme	750	0.29	2590
Solanum dubium enzyme	880	0.35	2490
Papain	216	0.59	367

High ratio indicates the suitability for cheese making as rennet substitute and/or additives

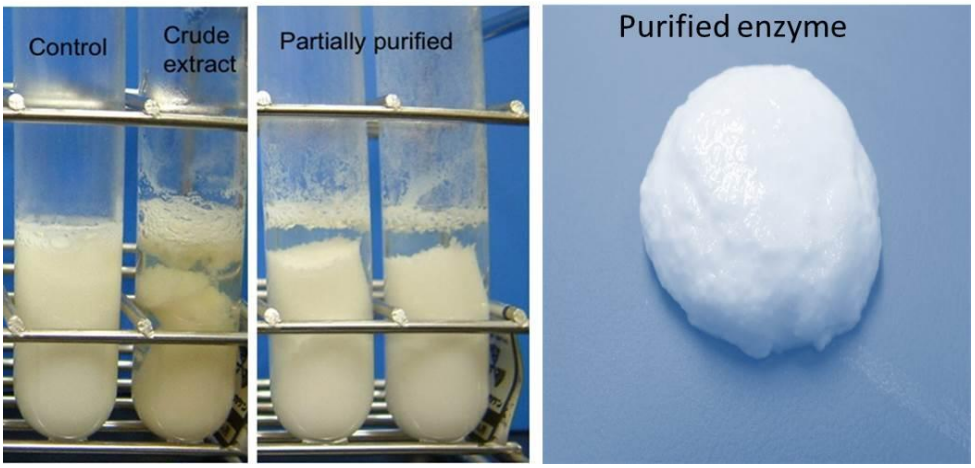

Fig. 6. Coagulation of skimmed milk by crude and partially and fully purified enzyme from *S. dubium* seeds

When a potential rennet substitute is considered, it is of most importance to adequately evaluate the degradation patterns of the caseins because of their effects on yield, consistency, and flavour of the final cheese (Fox, 1989). Hydrolysis of bovine casein and its components (α_s-, β-, and κ-caseins) were monitored by SDS-PAGE. *S. dubium* enzyme hydrolyzed bovine whole casein generated peptides, with amount progressively increasing with time (Fig. 7). Bovine whole casein was hydrolyzed into about six bands (marked as A, B, C, D, E, and F). Band A appeared after 5 min hydrolysis and thereafter disappeared after 6 h during hydrolysis. Bands B, C, and D appeared as early as 5 min after hydrolysis starts and their intensity increased as reaction time elapsed. Band C remained unchanged even after 24 h and was found to have a molecular mass of 16 kDa which is likely to be para-k-casein. In bovine whole casein, it was also observed that β-casein was completely degraded during 3 h incubation, while complete degradation of α_s-casein occurred after 6 h. This result revealed that β-casein is most susceptible to hydrolysis by *S. dubium* enzyme than α_s-casein. Hydrolysis of bovine whole casein by *S. dubium* enzyme was found to produce a number of bands corresponding to large peptide fragment. Similar studies found that milk-clotting enzymes of *Albizia julibrissin*, *Euonymus sieboldianus*, and *Celastrus orbiculatus* are primarily responsible for the breakdown of caseins to large peptide fragments (Otani *et al*., 1991). The results obtained in this study revealed that bovine α-caseins were less susceptible to the action of *S. dubium* protease than bovine β-casein. This may be an advantage of this enzyme to be used as a coagulant in the production of soft-flavored cheese as most of the coagulant used until now hydrolyzed bovine α-casein faster than β-casein (Trujillo *et al*., 1997; Tavaria *et al*., 1997; Jiang *et al*., 2007).

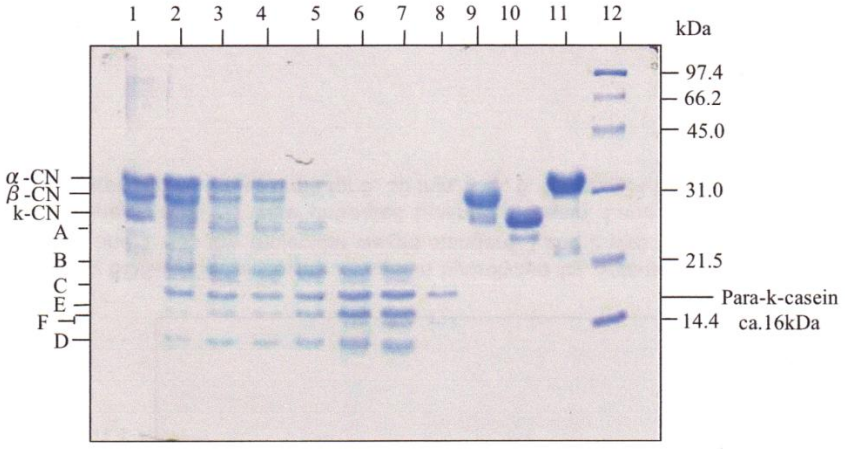

Fig. 7. SDS-PAGE pattern of bovine casein hydrolyzed by *Solanum dubium* enzyme. Lanes 1, 9, 10 and 11, unhydrolyzed whole bovine casein, α-, β-, and κ-caseins, respectively; lane 2-8, hydrolyzed whole bovine casein for 5, 30 min, 1, 3, 6, 12 and 24 h, respectively; lane 12 molecular weight marker.

CONCLUSION

Compared to other purification procedures, we succeeded in developing a simple purification procedure. The procedure is economic and, combined with the availability of plant seeds, could possibly be used for large-scale production of the enzyme, allowing a broad study of its various aspects and hence probable applications. Furthermore, the purified enzyme hydrolyzes casein fractions vary efficiently at different conditions. The high degree of hydrolysis on casein, in particular on β-casein, and the high milk clotting activity and high clotting activity/ proteolytic activity ratio of the isolated enzyme could be useful in the dairy industry both for milk clotting, as an alternative or with calf rennet, and for the acceleration of cheese ripening to reduce the time and costs of storage and maturation. Moreover, the high stability of the purified enzyme under various conditions, in accordance with the availability of raw materials, in addition to its high milk-clotting ability, could therefore pave the way for its use in the cheese industry as well as other food and biotechnological industries.

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